Action of Dissolved Substances upon the Autofermentation of Yeast.

By Arthur Harden, F.R.S., and Sydney G. Paine.

(Received October 17,—Read December 7, 1911.)

(From the Biochemical Department, Lister Institute.)

During experiments upon the permeability of the yeast-cell it was found that, when yeast was immersed in a molar solution of sodium chloride, and allowed to stand at air temperature, the amount of gas produced by autofermentation was considerably greater than that given by a water control.

The production of carbon dioxide by autofermentation of yeast is brought about by the action of at least two enzymes. The reserve material of the cell, for the most part glycogen, is first converted by a glycogenase into a sugar, which in turn is fermented by zymase with the production of alcohol and carbon dioxide. As the rate of autofermentation is considerably less than that produced by the same yeast in presence of excess of sugar, it follows that the rate of autofermentation is controlled by the rate of production of sugar within the cell, in other words, by the rate of action of the glycogenase. An increase in the rate of autofermentation, therefore, indicates greater activity of this enzyme within the cell. In order to investigate the action of solutions of various salts upon the rate of autofermentation of yeast, this was ascertained by measuring the volume of carbon dioxide evolved during successive intervals of time by means of the apparatus described by Harden, Thompson, and Young (1). The yeast employed was prepared from top-yeast as obtained from the brewery by pressing out the wort in a small hand press, it having been demonstrated (2) that practically the whole of the interstitial liquid can be removed in this way. A certain weight of such pressed yeast was carefully weighed into each of the fermentation flasks, and treated with a certain volume of the various liquids under experiment, controls being made with water. The liquids were saturated with carbon dioxide at 25°, the temperature of the water-bath.

1. *Effect of Sodium Chloride and other Salts upon the Autofermentation of Yeast.*

When yeast was immersed in molar sodium chloride solution the rates of evolution of gas during the first six successive intervals of 20 minutes

were 10·6, 8·4, 6·6, 4·9, 4·8, 4·8 c.c., as against 4·9, 4·2, 3·3, 2·9, 2·8, 2·7 c.c. when the same weight of yeast was immersed in water. In the former case fermentation practically came to an end after six hours, at which time 60 c.c. of gas had been collected as against 31 c.c. from the water control (Table I). In the latter case evolution of gas continued steadily until, after about 60 hours, the volume of gas was identical with that from the sodium chloride experiment.

Table I.—Effect of Sodium Chloride upon the Autofermentation of Yeast.

Time, in hours.	Cubic centimetres of carbon dioxide evolved by 3 grm. of yeast and 20 c.c. of solution.	
	Sodium chloride, molar.	Water control.
1	25·6	12·4
2	40·1	20·8
3	49·6	24·6
4	55·7	27·4
5	58·8	29·4
6	59·7	31·2
24	65·0	49·5
48	67·0	61·5
64	67·5	67·5

This experiment shows that, under the influence of molar sodium chloride, the whole of the fermentable material was decomposed in one-tenth of the time required by the water control.

Experiments were next made in order to determine the optimum concentration of this substance, which would give a maximum rate of autofermentation at the temperature employed.

Table II.—Effect of Varying Concentrations of Sodium Chloride.

No.	Cubic centimetres of carbon dioxide evolved during the first hour from 4 grm. of yeast + 10 c.c. solution											
	Water.	NaCl. 0·5 M.	NaCl. 0·6 M.	NaCl. 0·7 M.	NaCl. 0·8 M.	NaCl. 0·9 M.	NaCl. 1·0 M.	NaCl. 1·1 M.	NaCl. 1·2 M.	NaCl. 1·5 M.	NaCl. 1·7 M.	NaCl. 2 M.
42	21·7	—	—	—	—	—	82·0	—	68·5	57·7	42·5	42·5
47	—	35·6	37·4	41·7	43·1	44·1	42·9	—	—	—	—	—
48	—	—	—	49·0	50·6	50·1	44·7	44·2	41·9	—	—	—
48A	7·5	19·5	—	24·5	—	24·7	27·2	23·6	—	22·2	—	12·3

These results indicate that the optimum concentration varies slightly for different samples of yeast, but that it approximates to molar; moreover, very

slight difference is observable in the effect of concentrations ranging from 0·7 to 1·1 molar.

Experiments made with other salts showed that the phenomenon described for sodium chloride is a general one for all salts, both of inorganic and organic acids. The following salts were all found to give positive results: Chlorides of sodium, potassium, lithium, ammonium, magnesium, calcium, and barium; sulphates of sodium, potassium, ammonium, and magnesium; sodium salts of phosphoric, hexosephosphoric, arsenic, acetic, malic, citric, lactic, pyruvic, and glyceric acids.

With the salts of organic acids, the possibility exists that these may themselves be the source of the carbon dioxide. Neubauer (3) and Neuberg, Hildesheimer, Tir, and Karczag (4, 5, 6) have, in fact, stated that some races of yeast are capable of producing carbon dioxide from salts of lactic, glyceric, pyruvic, oxalacetic, and many other acids. As this phenomenon is accompanied by the disappearance of the acid in question, it can readily be distinguished from that which forms the subject of the present paper.

2. *The Nature of the Effect Produced by Salts on the Autofermentation of Yeast.*

It seemed advisable at the outset to ascertain experimentally if the increase in the rate of gas production were actually due to stimulation of the glycogenase, as was to be expected, or of the zymase. The sugar fermentation of 1 grm. of yeast immersed in molar sodium chloride gave only 1·7 c.c. of carbon dioxide per five minutes, as against 4·1 c.c. in the case of a water control. The action of the zymase is therefore inhibited rather than enhanced by this treatment. The increase in the rate of autofermentation would accordingly seem to result from a more efficient working of the glycogenase.

This might be due to one or more of the following causes:—

(1) To some specific action of the salt employed.

(2) To a concentration within the cell by removal of water as a result of plasmolysis.

(3) To removal from the cell of some substance or complex which has an inhibitory or controlling action upon the rate of glycogen fermentation.

(4) To disorganisation of the cell, whereby the factor controlling the access of enzyme to glycogen is in some way modified.

(5) To "hormone" action of the substance on the lines suggested by H. E. and E. F. Armstrong.

(1) *Specific Action.*—In order that a specific action should be exerted, it is essential that the agent should be capable of entering the cell. As regards

this question, in an earlier work* the conclusion was reached that most salts are probably not capable of penetrating beyond the outer layers of the cytoplasm. This would render any specific action upon the enzyme very doubtful. Moreover, it is improbable that so many different salts should exert a similar effect. Further, such action, if exerted in the cell, should also be exhibited in the contents after removal from the cell. The following table shows the result of addition of salt to yeast-juice both in presence and absence of added sugar :—

Table III.—Effect of Sodium Chloride upon Fermentation by Yeast-juice.

	Cubic centimetres of carbon dioxide evolved by 25 c.c. of yeast-juice in 18 hours.				
	Control.	+ 0·14 grm. NaCl.	+ 0·36 grm. NaCl.	+ 0·72 grm. NaCl.	+ 1·45 grm. NaCl.*
Sugar free.....	35·3	28·0	18·2	8·2	2·2
+ 1 grm. glucose...	55·9	42·2	29·8	14·5	3·1

* Molar concentration.

These numbers prove that the autofermentation is diminished in practically the same proportion as the sugar fermentation, and they afford no evidence of acceleration of the action of the glycogenase.

Very similar results were obtained with zymin.

Table IV.—Effect of Sodium Chloride upon Fermentation by Zymin.

	Cubic centimetres of carbon dioxide evolved by 5 grm. zymin + 20 c.c. solution in 5 hours.			
	Water.	M/10 NaCl.	M/4 NaCl.	M/2 NaCl.
Sugar free	77·2	64·0	51·7	32·4
+ 1 grm. glucose	173·2	162·4	136·2	83·5

It follows from these experiments that the direct action of salt upon the enzymes of yeast is that of an inhibitant, and that the acceleration of the autofermentation of yeast by salt cannot be due to a specific effect of the latter. This, however, does not exclude the possibility that certain substances which accelerate the action of yeast-juice and zymin may also exert a specific effect upon the autofermentation.

(2) *Plasmolysis of the Cell.*—It has been demonstrated by Paine that with molar concentration of sodium chloride strong plasmolysis occurs, while decimolar solution produces no such result. The effect of these concentrations upon the autofermentation of yeast is shown in the following table :—

* Paine, *loc. cit.*

Table V.—Showing Effect of Molar and Decimolar Solutions of Sodium Chloride.

Time, in hours.	Cubic centimetres of carbon dioxide evolved from 5 grm. yeast and 20 c.c. of solution.		
	NaCl, molar.	NaCl, decimolar.	Water control.
1	37·5	11·2	12·6
2	57·4	17·4	18·2
3	66·6	21·4	21·5
4	70·8	23·9	23·8
5	71·7	26·1	26·1

It follows that sodium chloride solution is without influence upon the auto-fermentation when the concentration is so low as to produce no plasmolysis of the yeast.

Experiments were, therefore, made to determine the effect of iso-osmotic solutions of various substances, which had all been found to produce plasmolysis in a similar manner to sodium chloride. The osmotic coefficients were taken from the tables given in Pfeffer's 'Physiology of Plants,' and in some cases the freezing points of the solutions were determined. The results are given in the following tables :—

Table VI.—Effect of Iso-osmotic Solutions of Salts.

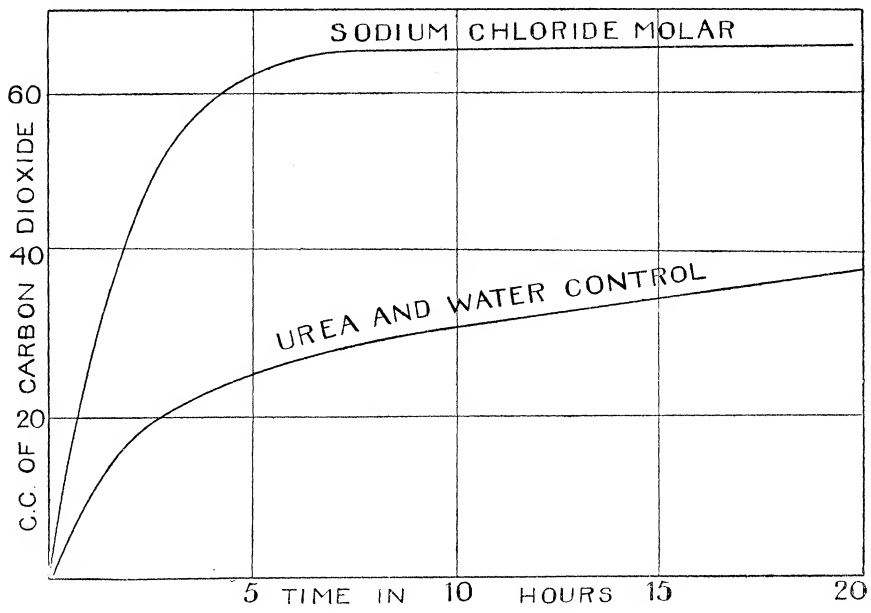
No. of expt.....	82. A.	B.	C.	D.	83. A.	B.	C.	D.
Details.....	10 grm. pressed yeast + 20 c.c. of solution.							
Substance employed	NaCl	K_2HPO_4	$CaCl_2$	Water control	NaCl	K_2SO_4	Mannitol	Water control
Concentration...	Molar	13 grm. 100 c.c.	8·3 grm. 100 c.c.	—	½ molar	6·5 grm. 100 c.c.	13·5 grm. 100 c.c.	—
Time, in hours.	Cubic centimetres of carbon dioxide.				Cubic centimetres of carbon dioxide.			
0	—	—	—	—	—	—	—	—
0·5	18·3	19·6	19·5	10·5	14·6	13·3	12·7	10·5
1·0	31·8	33·3	33·5	16·4	24·0	22·0	21·0	16·4
1·5	42·1	42·7	43·3	20·9	30·8	28·0	28·2	20·9
2·0	49·3	49·3	50·5	24·8	36·0	32·7	31·8	24·8
2·5	53·6	53·3	54·9	27·8	39·2	35·5	34·6	27·8
3·0	57·3	56·5	58·5	30·8	41·7	38·0	36·8	30·8
4·5	62·0	61·2	64·3	36·8	45·7	43·0	41·5	36·8

Table VII.—Effect of Solutions Iso-osmotic with 0·5 Molar Potassium Nitrate.

No. 84	10 grm. pressed yeast + 20 c.c. solution.					
Substance.....	KNO_3	$MgSO_4$	$BaCl_2$	Mannitol.	Glycerol.	Water.
Concentration.....	5·05 grm. 100 c.c.	18·45 grm. 100 c.c.	9·1 grm. 100 c.c.	13·65 grm. 100 c.c.	6·95 grm. 100 c.c.	—
Depression of freezing-point	1·46°	1·51°	1·80°	1·57°	1·52°	—
Time.	Cubic centimetres of carbon dioxide.					
12.45	—	—	—	—	—	—
1.15	29·5	27·0	30·1	29·2	27·6	19·0
1.45	54·3	50·0	57·8	56·4	50·0	31·6
2.15	75·8	70·0	80·9	78·5	66·6	41·0

These experiments point very strongly to the removal of water from the cell as the essential factor, since it is seen that, when substances which cause plasmolysis are employed, solutions of equal osmotic pressure produce an equal degree of acceleration.

In order to obtain convincing proof of this, it was necessary to find some



substance which would produce no plasmolysis of yeast even in concentrated solution, and to show that it would not cause acceleration. In earlier experiments urea was found to produce no plasmolysis at molar concentration. The determination of the effect of this substance upon the rate of autofermentation was therefore of first importance. In one experiment molar urea was compared with molar sodium chloride and water. The urea was found to be without influence, as shown in the curves (p. 453).

In the following experiment (No. 85) the effects of isotonic solutions of urea, sodium chloride, and potassium nitrate were compared.

Table VIII.—Effect of Urea Solutions.

No. 85.	10 grm. yeast + 20 c.c. solution.				
	NaCl	Urea	KNO_3	Urea	Water
Concentration.....	5·85 grm. 100 c.c. = molar	9·0 grm. 100 c.c.	5·05 grm. 100 c.c. = 0·5 molar	4·5 100 c.c.	—
Depression of freezing point	—	—	1·46°	1·42°	—
Time.	Cubic centimetres of carbon dioxide.				
0·0	—	—	—	—	—
0·5	34·0	17·0	21·3	16·5	17·0
1·0	65·0	29·2	46·7	29·4	29·3
1·5	86·5	34·5	60·3	35·5	35·5

Urea is thus seen to be without influence upon the rate of autofermentation, although, as shown by the depression of the freezing-point, the solutions of this substance were isotonic with the corresponding salt controls. The fact that plasmolysis of the cells is not produced by urea solutions would seem to indicate that this substance can penetrate freely through the cytoplasm of the yeast cell. An experiment was made to investigate this point, the method described by Paine (2) being employed; 100 grm. of yeast were suspended in 100 grm. of molar urea solution, allowed to stand 20 hours at a temperature approximating to zero, and the distribution of urea determined (Table IX).

Urea is thus seen to penetrate readily into the cells, the factor K representing the coefficient of diffusion being of the same order as that obtained for alcohol, namely, 0·85 to 0·87. Although urea enters the cells it is without influence upon the rate of autofermentation.

Table IX.—Showing Diffusion of Urea into the Yeast-cell.

	Initial yeast.	Initial liquid.	Final yeast.	Final liquid.	P = grm. urea per 100 grm. water within the cells.	P_1 = grm. urea per 100 grm. water outside the cells.	$K = P/P_1$.
	grm.	grm.	grm.	grm.			
Solids other than urea	32·50	—	31·61	0·85			
Urea	—	5·94	2·50	3·45			
Water	67·50	94·06	72·49	89·10			
Total weight.....	100·00	100·00	106·60	93·40	3·43	3·87	0·89

Removal of Water by Partial Drying.—If the acceleration of the enzymic activity were due simply to concentration within the cell, removal of water by drying would be expected to produce the same result as removal of water by plasmolysis. In order to investigate this 10 grm. of pressed yeast which had been passed through a 3 mm. sieve were placed in a fermentation flask and subjected to a current of air for 20 minutes. This flask and a control were then connected with the gas-measuring apparatus and warmed in the water-bath at 25°. The rate of autofermentation was considerably increased by this simple method of removing water.

Table X.—Effect of Partial Drying by Air.

Time, in mins.	Cubic centimetres of carbon dioxide given by 10 grm. yeast.	
	After 20 minutes blow.	Control.
15	14·5	4·3
30	27·5	8·9
45	36·4	13·3
65	43·6	18·4
85	47·8	23·4

In another experiment three lots of 10 grm. of pressed yeast were weighed out, of which B and C were dried in a vacuum desiccator for two and four hours respectively, whereby B lost 2 grm. and C 3·2 grm. of water. The rate of autofermentation of these samples was compared against A as control.

Table XI.—Effect of Partial Desiccation *in Vacuo*.

Time.	Cubic centimetres of carbon dioxide yielded per hour by—		
	A. 10 grm. yeast. Control.	B. 10 grm. yeast dried 2 hours.	C. 10 grm. yeast dried 4 hours.
1st hour	36·0	46·1	55·5
2nd „	15·3	17·2	39·3
3rd „	14·1	14·7	38·7
4th „	12·9	14·8	32·4
5th „	13·7	14·7	22·4
24 hours (total)	281·6	248·3	234·9

In this experiment a loss of 3·2 grm. of water from 10 grm. of yeast, equal to approximately half the water content of the cells, had the effect of more than doubling the rate of autofermentation.

(3) The possibility of the removal from the cell of some inhibitory or controlling substance during plasmolysis is negatived by these last experiments, wherein the increase of autofermentation was produced under conditions which render such removal impossible unless the substance be a volatile liquid.

(4) The disorganisation of the cell, possibly by the disintegration of a material membrane or network, has been adduced as the cause of some of the effects of anæsthetics on the living cell [Overton (7), Lepeschkin (8), Hans Meyer (9)], and it is not impossible that in certain instances this phenomenon plays some part in the acceleration of the autofermentation of yeast. This possibility is specially present in the case of a substance like toluene, which exerts an anæsthetic effect upon yeast.

The following experiment is typical of many; 10 grm. yeast were mixed with (*a*) 25 c.c. water, (*b*) 25 c.c. water and 5 c.c. toluene, well shaken, and incubated at 25°:—

Table XII.—Effect of Toluene on the Autofermentation of Yeast.

Time.	Cubic centimetres of carbon dioxide.			
	a. Water.		*b*. Water + toluene.	
	Total.	Rate per 10 mins.	Total.	Rate per 10 mins.
10 mins.	3	3	8·5	8·5
20 „	5·3	2·3	15·5	7·0
30 „	7·6	2·3	22·7	7·2
40 „	9·4	1·6	29	6·3
50 „	11·3	1·9	35·9	6·9
3 hrs.	21·5	—	86·1	—

Other instances of the same effect are the following, all of which refer to 10 grm. of yeast:—

Table XIII.—Effect of Toluene.

Date.	Time, in hours.	Water alone.	Water + toluene.
3.2.08	1	4·2	29·5
10.9.09	2·5	6·3	30·4
17.9.09	2	6·9	34·5
17.10.07	4	48·9	80·7
20.10.07	5	28	97·6

As in the case of salt solutions the rate slowed down comparatively soon, owing to exhaustion of the fermentable material. The effect is not due to a specific action on the enzymes, since toluene has either no effect or a slight inhibitory effect on the autofermentation of yeast-juice, as is shown by the following result: 25 c.c. of yeast-juice in three hours gave 40·3 c.c. of CO_2; in presence of 5 c.c. of toluene the same volume of yeast-juice gave 34 c.c.

It is, however, not impossible that this result may be explicable on the ground of plasmolysis. In spite of the small solubility of toluene in water a considerable degree of plasmolysis is observed when yeast is shaken with water and excess of toluene. Further experiments on this point are in progress.

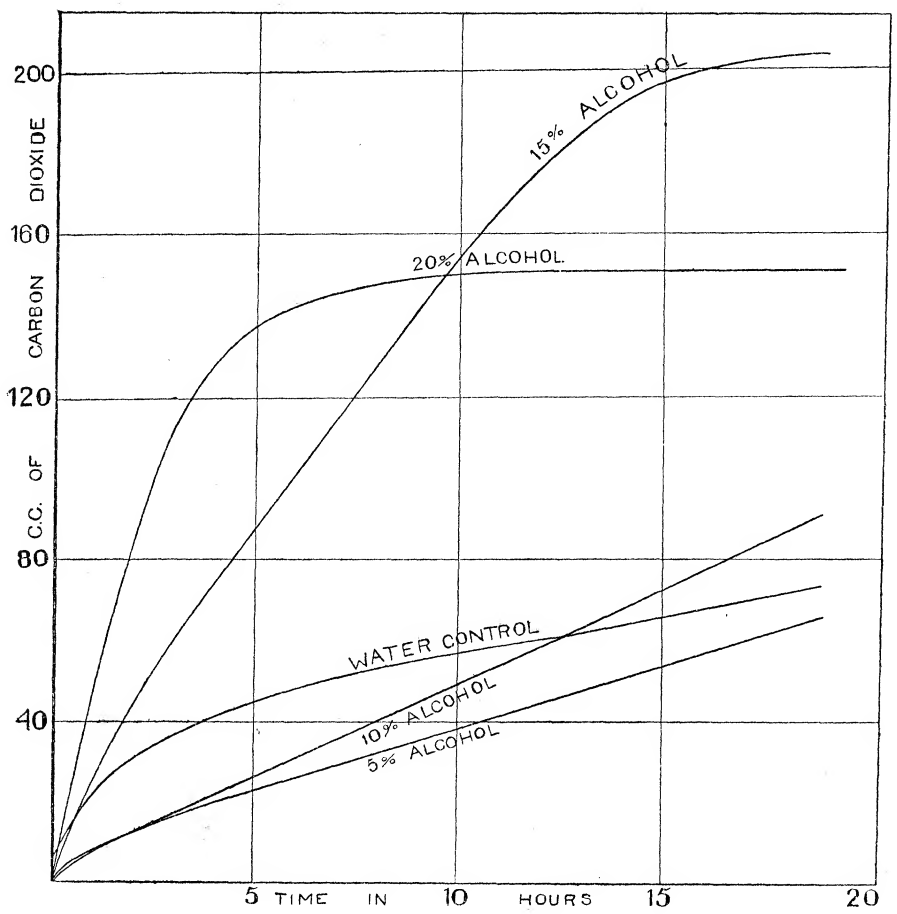
(5) With regard to the possibility that the foregoing changes may be ultimately due to the action of hormones in the manner suggested by H. E. and E. F. Armstrong (7) no very definite conclusion can be drawn. The action of toluene on yeast undoubtedly presents the closest analogy to that which it exerts on the Aucuba leaf, and it cannot be denied that the various salts employed do penetrate at all events into the outer layers of the yeast cell. Several of the phenomena, however, appeared to be difficult to explain in this way, especially the lack of action of a substance like urea, which penetrates the cell, and the causation of the phenomenon by simple drying. In any case the acceleration caused by salts is accompanied by concentration of the cell contents, so that dilution cannot in these instances be the effective cause, as suggested by Armstrong* for the phenomenon observed by him.

3. *Effect of Alcohol on Autofermentation.*

The plasmolysing effect on yeast of solutions of alcohol was found to be practically absent from concentrations up to 10 per cent. (rather more than

* *Loc. cit.*

twice molar), but above this concentration plasmolysis became well marked. The influence of alcohol solutions on the autofermentation of yeast is shown by the following curves, which apply to 10 grm. of yeast:—



Concentrations of alcohol which plasmolyse the cells produce a considerable increase in the rate of autofermentation. With 20 per cent. the action of the enzyme almost came to an end after about seven hours, at which time 147 c.c. of gas had been collected as against 52 c.c. from the water control. The weaker concentrations of alcohol at first produced an inhibitory effect upon the rate. After a short time, however, the rate increased, and then slightly exceeded that of the water control.

Eventually, after eight days, the volume of gas yielded from each, with the exception of that in presence of 20 per cent. alcohol, was practically identical and approximately equal to 200 c.c.

The behaviour of alcohol, therefore, is in accord with that of urea, although the effect is not quite so simple.

Summary.

1. All dissolved substances which plasmolyse the yeast-cell also cause a large increase in the rate of autofermentation.

2. Substances such as urea, which even in concentrated solution do not produce plasmolysis, have no accelerating effect.

3. Toluene produces a similar effect to concentrated salt solutions.

4. The effect produced by salts is probably a direct result of the concentration of the cell contents due to plasmolysis, but in the case of toluene it is possible that some other factor (such as disorganisation of the cell, or hormone action) is concerned.

REFERENCES.

1. Harden, Thompson, and Young, 'Biochem. Journ.,' 1910, vol. 5, p. 230.
2. Paine, 'Roy. Soc. Proc.' (in press).
3. Neubauer and Fromherz, 'Zeitschr. f. Physiol. Chem.,' 1911, vol. 70, p. 326.
4. Neuberg and Hildesheimer, 'Biochem. Zeitschr.,' 1911, vol. 31, p. 170.
5. Neuberg and Tir, *ibid.*, 1911, vol. 32, p. 323.
6. Neuberg and Karczag, *ibid.*, 1911, vol. 36, p. 60.
7. Overton, 'Jahrb. f. Wiss. Bot.,' 1900, vol. 34, p. 670.
8. Lepeschkin, 'Ber. Deutsch. Bot. Ges.,' 1911, vol. 28, p. 383, and vol. 29, p. 349.
9. Meyer, Hans, 'Report Int. Cong. Applied Chemistry,' 1909, IVA, vol. 2, p. 37.
10. Armstrong, H. E. and E. F., 'Roy. Soc. Proc.,' B, 1910, vol. 82, p. 588.